Isolation and Characterization of the High Molecular Weight Brown Colorant of Maple Sirup

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The colorant from maple sirup was isolated and separated into two molecular species by gel filtration. The molecular weight of the largest species was 7000 to 45,000 as determined by ultracentrifugation. Qualitative chemical tests indicated the presence of carbohydrate and carboxyl groups, but were negative for protein and aromaticity. The absorption spectra in the near-ultraviolet and visible regions resembled those of typical carbohydrate solutions. Hydrolysis produced a large number of carbohydrates, two carboxylic acids, and small amounts of amino acids. The composition indicated that the colorant was derived from alkaline degradation products of sugars. A formula for the high molecular weight colorant is suggested.

Color is the dominant grade and price-determining factor for maple sirup, with lighter sirups generally preferred. Control of the degree of color formation and the grade of sirup produced requires fundamental knowledge of the nature of the maple sirup colorant and the mechanism of its formation. This paper describes the isolation and preliminary characterization of the high molecular weight colorant and discusses a possible mechanism of formation.

The empirical formula of a maple sirup colorant isolated by ion exchange procedures has been reported as $C_{18}H_{27}O_{12}N$ by Underwood, Willits, and Lento (*21*). The nitrogen content of the colorant, 3.1%, could be either an integral part of the material and thus evidence of a Maillard-type reaction (*12*) or a contaminant resulting from degradation of the ion-exchange resin (*7*). A search was therefore made for an effective procedure for isolating maple colorant from the sugar, without introduction of extraneous nitrogen. The problem was complicated by the extremely high ratio of sucrose to colorant and the similarity in behavior of sucrose and colorant toward conventional methods of separation such as dialysis and solvent extraction.

In a previous paper (*18*) the authors described a method of gel filtration used to separate caramel colorant from sugar and unassociated ash, whereby it was possible to separate the few parts per million of colorant from sirups containing 65% sugar and as much as 2% salt and other nonsugar solids. This paper describes the successful application of this procedure to the isolation and purification of the colorant from maple sirup.

Experimental and Results

Maple Sirup Marc. This marc was prepared from medium amber maple sirup, U. S. Grade A, by removing the flavor components by exhaustive chloroform extraction. This procedure left the colorant, sugars, and salts in the aqueous solution or marc (*21*).

Isolation Procedure. Gel filtration was used to separate the small molecular weight substances of the marc (sugar, salts, etc.) from the large colorant molecules. This procedure resembles dialysis by separating molecules on the basis of size, but instead of membranes uses porous dextran particles in which the dimensions of the pores are diminished by crosspolymerizing with methylene units. This limits all molecules having dimensions greater than the pores to the interparticular liquid while smaller molecules whose movements are not hindered are uniformly distributed in both the intra- and interparticular liquids. This effect was utilized in both a nonflowing procedure for concentration of the colorant and a column procedure for final purification.

The nonflowing or batch procedure consisted of adding marc diluted to 40° Brix to dry dextran gel. As the particles hydrated, the low molecular weight substances were uniformly distributed in both liquid phases while the high molecular weight polymeric colorant was concentrated in the interparticular liquid (*10*). Separation of this liquid phase by filtration resulted in a solution containing virtually all of the high molecular weight material.

The dextran was prepared for this procedure by first removing the fines from 800 grams of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., New York, N. Y.), coarse grade, by repeatedly suspending the material in water and decanting the supernatant liquid after the coarse particles had settled. After the final decantation the Sephadex was rinsed successively with 95% ethanol and absolute ethanol, and dried by heating at 105° C. for 1 hour. The dried Sephadex (approximately 780 grams) was added to 4000 ml. of the diluted sirup marc. The suspension was stirred 30 minutes to permit complete equilibration and then filtered using mild suction. The final volume of the filtrate, including two 100-ml. rinses, was 2150 ml.

This nonflowing procedure was repeated until 76 liters of sirup marc had been treated. The filtrates were combined (total volume approximately 41 liters) and the concentration was repeated, treating successive 4000-ml. aliquots by the nonflowing procedure until all of the filtrate had been reconcentrated. The entire procedure was repeated five times, which resulted in all of the relatively pure colorant being concentrated in 4000 ml. The Sephadex showed no evidence of physical breakdown or change of properties.

The purification of the color concentrate consisted of adding 200-ml. portions of this concentrate to columns 5 × 150 cm. I.D. packed with 450 grams of Sephadex G-25, medium grade, from which the fines had been removed as previously described. The large colorant molecules present only in the interparticular liquid passed through the column on washing with distilled water and emerged as a discrete band before the smaller colorant molecules, which were retarded by diffusing into the gel and appeared in the eluate as a later band together with the sugar and salt molecules. Fractionation of the eluate as it emerged from the column permitted the separation of high molecular weight colorant, the first band to be eluted, from the rest of the material.

The Sephadex columns were tested in preliminary experiments to establish the relative contribution of the high and low molecular weight fractions to the colorant and their recovery in the eluates. Two hundred-milliliter portions of marc diluted to 40° Brix were added to the Sephadex column and washed through the column using water having a 4-foot hydrostatic pressure. The eluate was collected in 20-ml. fractions. Each fraction was examined for color by visual comparison with MacAdam glass

color standards (*3*) and aliquots of each fraction were tested for sugars by the anthrone reagent.

The coloration was separated by the Sephadex column into two bands which appeared in the eluate between fraction 26 (520 ml.) and fraction 110 (2200 ml.). The lower band of higher molecular weight colorant was most concentrated in eluate fraction 45 (900 ml.) and the upper band which contained the low molecular weight colorant together with the sugars and salts was most concentrated in eluate fraction 90 (1800 ml.). Sugars were first detected in eluate fraction 60 and were present in all succeeding fractions. This indicated that the fractions containing the higher molecular weight colorant (the lower band) were free of sugars and other low molecular weight contaminants, while the fractions containing lower molecular weight colorant (the upper band) contained sugars and other low molecular weight material.

The eluate fractions of each of the two colored bands were combined and concentrated to 200 ml. Comparison of the color intensity by means of a Dubosq colorimeter disclosed that the solution containing the higher molecular weight colorant contributed 25% of the color while the solution containing the lower molecular weight color contributed 75% of the original color. To test the recovery of colorant from the Sephadex the two 200-ml. solutions were combined and concentrated to 200 ml. The color concentration equaled that of the original solution. This indicated complete recovery of color from the Sephadex columns. All concentrations were made using a rotary vacuum evaporator at temperatures below 25° C.

This column purification procedure was used to isolate the high molecular weight colorant in the concentrate from the nonflow concentration procedure described previously, treating 200-ml. portions each time. All of the eluates of high molecular weight colorant were combined, concentrated to 400 ml., and lyophilized. This gave 2.54 grams of high molecular weight colorant.

Lyophilization produced no detectable alteration in the properties of the colorant. It was still water-soluble, produced the same coloration when redissolved, and when passed through a gel filtration column, was eluted in those fractions of the eluate typical of the original material. There was no loss in color of the lyophilized powder.

Physical and Chemical Properties. The lyophilized powdered colorant, which was light brown, had moderate heat and storage stability. It withstood boiling (pH 6.5) as long as 30 minutes and storage as long as a year under desiccation at room temperature without color change. The hygroscopicity of the powder resembled that of starch or cellulose, as it could be exposed to the atmosphere for considerable periods with only moderate increase in weight and retained its powder character. The material was soluble in water but insoluble in the usual organic solvents. The adsorption spectra in regions between 205 and 800 mμ of the original sirup and the high and low molecular weight colorant are given in Figure 1.

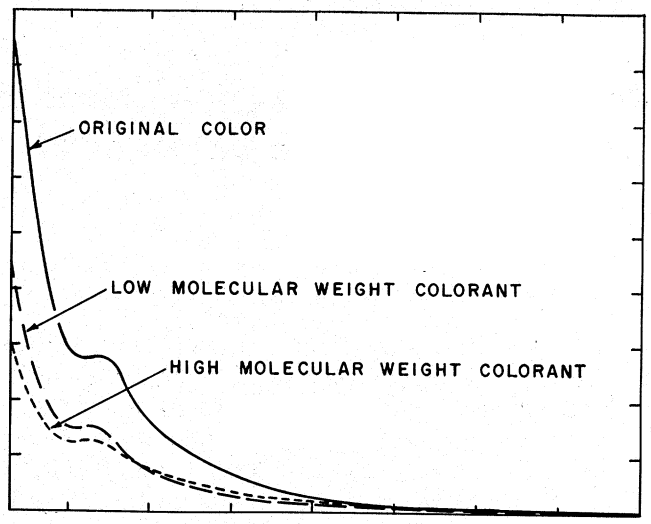

Figure 1. Absorption spectra of maple sirup and the high and low molecular weight colorant extracted from it

Except for a small peak at 270 mμ present in all three solutions, the spectral curves of the three materials are similar, with strong absorption at 205 mμ decreasing asymptotically to very low absorption at 800 mμ. When the solution containing high molecular weight colorant was made basic, this peak shifted to 290 and two new peaks appeared at 244 and 345 mμ.

Ultracentrifugation sedimentation curves of the high molecular weight colorant showed a small peak, representing 15% of the colorant, corresponding to a molecular weight of 12,000. The curve indicated that the remainder of the colorant was uniformly distributed between 7000 and 45,000, with the median molecular weight between 10,000 and 12,000.

Qualitative chemical tests were made on the isolated higher molecular weight colorant for classification and functional groups (Table I).

The tests for unsaturation were not conclusive, because of possible interference by other functional groups. The usual distinguishing criterion, evolution of gaseous HBr from carbon tetrachloride, could not be observed because of the limited solubility of colorant in the solvent.

For quantitative determination of carboxyl groups, a sample of the colorant was dissolved in water, the cations were removed by passing the solution through a Dowex-50 (H^+) column, and the solution was neutralized by potentiometric titration using standard alkali. A water blank was used as a control.

Table I. Qualitative Tests on High Molecular Weight Colorant for Classification and Functional Groups

Test	Result	Reference
Protein		
Millon	Neg.	(*8*)
Tetrabromophenol-phthalein, ethyl ester	Neg.	(*9*)
p-Dimethylamino-benzaldehyde	Neg.	(*9*)
Carbohydrate (general)		
Anthrone	Pos.[a]	(*8*)
Reducing sugars		
Tollens	Pos.	(*9*)
Triphenyltetrazolium	Pos.	(*9*)
Carbonyl groups		
2,4-Dinitrophenyl-hydrazine	Pos.	(*8*)
Semicarbazide	Pos.	(*17*)
Hydroxyl groups		
Periodate	Pos.	(*9*)
Acetyl chloride	Pos.[b]	(*8*)
Aromaticity		
Lieberman	Neg.	(*9*)
Millon	Neg.	(*9*)
Unsaturation		
Baeyer	Pos. (?)	(*8*)
Bromine/water	Pos. (?)	(*8*)
Phenolic groups		
Ceric nitrate	Neg.	(*8*)
Ferric chloride	Neg.	(*8*)
Ferricyanide	Neg.	(*8*)
Carboxyl groups		
Hydroxamate	Pos.	(*9*)

[a] Yellow-brown color.
[b] No alteration in color or odor, but paper chromatography indicated reaction.

The rate of pH change upon addition of base increased gradually from pH 3.68 to 6.4 with a rapid rate between 6.4 and 8.0 and then decreased gradually. The increase in alkali consumption over the control indicated a carboxyl content of 4.8%.

A portion of the colorant was further purified for elemental analysis by dissolving the dried colorant and subjecting it to five successive passes through the dextran column. The results of the

analysis performed after the fifth repurification are given in Table II. The metals were determined by arc spectroscopy.

Portions of both the original colorant and its acid hydrolyzate (described below) were spotted on Whatman No. 1 paper and examined by ascending paper chromatography with various solvents. After development, the papers were dried, viewed under visible and ultraviolet light, and subsequently sprayed with acid (*6*), amino acid (*15*), and carbohydrate (*1, 4, 14, 19*) indicators.

Paper chromatography of the original high molecular weight colorant with water as the developing solvent produced an elongated streak having an average R_f of 0.74. Addition of increasing amounts of *N,N*-dimethylformamide to the water diminished the movement but failed to separate the visible material into more than one area. Acidifying the media by adding acetic acid had no discernable effect on the movement.

The area containing visible material fluoresced under ultraviolet light and gave positive reactions with the carbohydrate and acid indicators, but no reaction with the amino acid indicator. These sprays also failed to indicate separation of material into more than one area.

Use of organic solvents such as methanol, acetone, and 1-butanol–acetic acid–water (4 : 1 : 5) caused no apparent movement of material other than a slight tendency to streak with the last solvent. However, when the chromatograms were dried and redeveloped with water, a considerable portion of the colorant had been rendered immobile, indicating denaturation of colorant by organic solvents.

Paper chromatography of an acid hydrolyzate, prepared by acidifying a 2% solution of high molecular weight colorant adjusted to 0.1*N* HCl and heating 24 hours on a steam bath, gave the information summarized in Table III. These conditions would give some hydrolysis of polypeptides, but would cause complete hydrolysis of *N*-glycosides and Schiff bases.

The 1-butanol–acetic acid–water solvent mixture was selected for use as it gave good separations and much literature is available on its use with different classes of compounds.

No movement of colored material was visible on the chromatogram but three well defined fluorescent zones with R_f values of 0.00, 0.21, and 0.49, respectively, were observed on exposure to ultraviolet radiation.

The acid indicator (bromophenol blue) revealed the presence of two acidic areas, a large region with an R_f of 0.27 and a much smaller region with an R_f of 0.61. The acids were not identified, but the R_f values differ considerably from those of malic and succinic acids (*5*).

The amino acid indicator (ninhydrin) revealed nine separate spots. Two-dimensional paper chromatography by the Levy and Chung (*11*) and Rockland and Underwood (*16*) procedures identified the different areas as aspartic acid, glutamic acid, serine, alanine, and traces of asparagine. Although no quantitative determinations were made, it was estimated that the trace amounts of serine and alanine were equal and that the concentration of each was approximately four times that of aspartic and glutamic acids, which in turn were equal. Three other very weak spots were tentatively identified as lysine, methionine or valine, and leucine or isoleucine.

The chromatograms of the acid hydrolyzate, when sprayed with the carbohydrate reagents ($AgNO_3/NH_3$, $AgNO_3$/NaOH, and aniline phosphate), produced a large number of stained areas, not all of which may represent carbohydrates, since these reagents are known to react with other classes of compounds. Atypical colors are indicated in the table. No areas were positively identified, but glucose and sucrose were definitely absent (*13*).

Table II. Elemental Analysis of High Molecular Weight Colorant

	%, Dry Basis
Elements	
C	40.39
H	6.43
N	1.18
Ash	4.49
O (diff.)	47.51
Metals	
Potassium	1.42
Calcium	1.97
Magnesium	0.742
Iron	0.105
Copper	0.023
Sodium	0.149
Boron	41 p.p.m.
Manganese, aluminum, silicon	Traces
Phosphorus	0.203

Discussion

The simplest empirical formula for the larger molecular species of the colorant that may be calculated from the elemental analysis on an ash-free basis is $(C_{40}H_{76}O_{35}N)_x$. The molecular weight of 7000 to 45,000 as determined by ultracentrifugation indicated that x may vary from 6 to 40, corresponding to molecular weights of 6780 and 45,200. The average molecular weight of this species, estimated at 10,000 to 12,000, would be reached when $x = 10$, corresponding to a molecular weight of 11,300.

The diminution of ash content of the colorant from 10.4 and 8.1% after the first and second repurification, down to 4.5% after the fifth repurification with the dextran, indicated a weak, probably carboxylic, bond between cations and colorant, while the relatively constant nitrogen content of 0.90, 1.17, and 1.18% after the first, second, and fifth repurifications, respectively, indicated that nitrogen was present as an integral part of the colorant. The nitrogenous compounds identified in the acid hydrolyzate —serine, alanine, aspartic acid, and glutamic acid—were present in the proportions 4 : 4 : 1 : 1, respectively. The aggregate composition of these amino acids comprises less than 1.18% of the colorant.

The following empirical formula of the larger species of the colorant was suggested on the basis of these data.

$$[\text{Cations}]^{+12}\ \underbrace{[C_{359}H_{710}O_{316}}_{\text{Unidentified}}\ \underbrace{(\text{—CONH—})_{10}\ C_{21}H_{40}O_4\ (CO_2)_{12}]^{-12}}_{\text{Amino acid residue}}$$

The peptide bond indicated in this formulation is considered the probable nexus between amino acids and colorant, although other types of bonds such as ester, ether, etc., are possible. However, the exact nature of the bond would have minor effect on the essentially saturated nature of the unidentified portion of the polymer.

In this formula, carboxyl groups from the amino acid residues comprised 4.7% of the colorant, which is close to the value (4.8%) obtained by potentiometric titration of solutions of higher molecular weight colorant acidified by passage through a Dowex 50-H^+ ion-exchange column. However, it is probable that the amino acids did not contribute all of the titratable acidity, as paper chromatography revealed the presence of carboxylic acids. Thus a portion of the amino acids could be present in the form of polypeptide chains instead of a simple amino acid–organic acid linkage. No evidence was found of nitrogen-containing materials other than the amino acids.

The unsaturated chromophores are in the essentially saturated carbohydrate (major) portion of the molecule. Since the analysis indicates only a small amount of unsaturation, the remainder of the carbohydrate portion of the molecule must be highly saturated, with most non-carboxylic oxygen present in either hydroxyl or ether groups. The oxygen content is lower than the content typical of most carbohydrates, which indicates that dehydrated intermediates such as acetol have condensed to form part of the polymer. Acetol, previously identified as an important component of the steam distillate of maple sirup (*20*), has been postulated as an intermediate in color formation during alkaline glucose degradation (*2*).

No direct evidence has been obtained on the mechanism of color formation in

Table III. R_f Values of Acid Hydrolyzate of High Molecular Weight Colorant with 1-Butanol–Acetic Acid–Water (4 : 1 : 5)

				Carbohydrates		
R_f	UV-Fluorescent	Acids–Bromophenol Blue (6)	Amino Acids–Ninhydrin (15)	$AgNO_3/NH_3$ (14)	$AgNO_3/NaOH$ (19)	Aniline phosphate (4)
0.00	0.00 (origin)[a]				0.00	0.00
0.10			0.06 (lysine)			0.07
			0.09 (asparagine)		0.10	
			0.15 (aspartic)	0.14 (white)	0.15 (yel.)	0.16 (yel.)
			0.18 (serine)	0.18 (white)		
0.20						
	0.21		(0.24 (glutamic)			
		0.27		0.26	0.24	0.26
0.30			0.32 (alanine)		0.38	
0.40			0.44 (methionine or			0.42
	0.49		valine)		0.50	
0.50						
0.60					0.57	
		0.61	0.61 (visible-colorless)			
			(UV-fluorescent)		0.63 (yel.)	0.63 (yel.)
			0.67 (leucine or iso-leucine)			
0.70						
0.80						0.81

[a] Visible coloration.

maple sirup during the present study. However, the carbohydrate nature of the colored material is consistent with the view that maple sirup coloration developed during the sap evaporation is formed by a mechanism resembling the one proposed by Berl and Feazel for color formation during alkaline glucose degradation (*2*). This mechanism involves rapid formation of trioses such as acetol and dihydroxyacetone from reducing sugars, polymerization of these fragments to form brown, water-soluble polymers, and eventual condensation and polymerization to form insoluble black material. Carbohydrate degradation would appear to be the major intermediate of maple sirup color, as Willits *et al.* (*22*) used model systems that simulated maple sap to show that the concentrations of the amino acids occurring in maple sap would not be sufficient to form the observed coloration. However, contribution to the total color from the Maillard reaction cannot be eliminated, since it is possible that the 1% nitrogen present is chemically bound in a small fraction of the colorant macromolecule.

These data indicated that the colorant polymers contain a very small amount of highly unsaturated linkages in the presence of a very large amount of highly saturated ones derived mainly from carbohydrates. The strongly alkaline nature of maple sap during the early stages of its evaporation favors the degradation of the simple sugars to trioses (*20*). The saturated character of the color isolate indicated that color formation had been halted at the polymerization stage, at which point the chromophore centers had been formed. Further reaction was arrested by the shift to neutrality that occurs during the final stage of the sap evaporation.

The maple colorant possessed an absorption peak at 270 mμ in acid solution that shifted to 290 mμ in alkaline solution, similar to the glucose chromogen formed in alkaline solution, which had an absorption peak at 265 mμ in acid solution and 295 mμ in alkaline solution. The smaller intensity in the maple colorant was probably due to less extensive reaction because of the milder conditions and shorter duration.

However, deepened color and eventual formation of insoluble precipitate in alkaline solutions of the isolated maple colorant indicated that further reaction of maple colorant under suitable conditions occurs and results in a final product resembling the final stage of the non-enzymatic, non-Maillard glucose "chromogen" reaction of Berl and Feazel.

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